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Composition and Biophysical Properties of Lipids in *Xenorhabdus nematophilus* and *Photorhabdus luminescens*, Symbiotic Bacteria Associated with Entomopathogenic Nematodes

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Primary and secondary forms of *Photorhabdus luminescens* Hm and *Xenorhabdus nematophilus* N2-4 were grown at 18 and 28°C for 24 to 96 h, and we made determinations of the fatty-acid compositions of total lipids and of the fluidity measured by 5-doxyl-stearic acid embedded in liposomes made from total lipids. The levels of the unsaturated fatty acids 16:1 and 18:1 (those with chain lengths of 16 or 18 and one double bond) generally were higher in primary-phase variants of *P. luminescens* grown at 18°C than in those grown at 28°C. Prolonged culture at 18°C caused the level of 18:1 to fall and reach that observed at 28°C. The ratio of saturated to unsaturated fatty acids rose with prolonged culture times in variants of each species at both phases. When grown at 18°C, the proportion of 16:1 in *X. nematophilus* was lower than in *P. luminescens*; the patterns of temperature-induced changes were similar in these species. *X. nematophilus* contained a greater percentage of short-chain fatty acids (i.e., with chain lengths of <14.0) than *P. luminescens*. Lipid liposomes from primary and secondary cultures of both bacterial species grown at 18°C were more ordered (i.e., less fluid) than those grown at 28°C. This result suggests the surprising absence of homeoviscous adaptation of membranes to temperature. Also, liposomes from primary cultures were more ordered than those from secondary cultures and membranes from primary cultures of *P. luminescens* were more ordered at both culture temperatures than membranes from *X. nematophilus*. The biological significance of the effect of growth conditions on membrane biophysical properties in these bacteria is discussed.

The infective developmental stage (the dauer juvenile) of the entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis* spp. introduces symbiotic bacteria (*Xenorhabdus nematophilus* and *Photorhabdus luminescens*, respectively) into insects, which are subsequently killed by bacterial infection. These bacteria exhibit a well-pronounced dimorphism. The primary-phase variants of these bacteria occur exclusively in the digestive tracts of the dauer juveniles (3) but transform into the so-called secondary forms under in vitro conditions. The nematodes accumulate the primary-phase variants even when they are propagated in a medium containing 80% secondary forms (1). There are characteristic biochemical differences between variants of the two phases with respect to the production of antibiotics (2, 19, 21) and pigments (1, 21, 22) and the secretion of enzymes (lipases, proteases, etc.) (1, 2, 5, 6, 22); the primary forms are more metabolically active than the secondary forms. The appearance of secondary-phase variants in culture medium leads to poor yields of infective juvenile nematodes (6, 13, 14). The factors controlling the transition from the primary- to the secondary-phase variant are unknown.

The lipid components of biological membranes are the barriers separating the cell from its environment while regulating the flux of materials and metabolites. Because the primary and secondary forms differ with respect to releasing certain metabolic products and because only the primary forms survive in the nematode digestive system, it seemed interesting to study the compositional and biophysical properties of membrane lipids isolated from these bacteria. Special attention was given to the effect of growth temperature on these parameters, because the nematode must survive under adverse thermal conditions which might influence symbiont-host relationships.

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MATERIALS AND METHODS

Culture of bacteria. The primary- and secondary-phase variants of *P. luminescens* Hm and *X. nematophilus* N2-4 were grown on indicator plates of NBTA (5 g of Bacto Peptone, 3 g of beef extract, 15 g of Bacto agar [Difco], 25 mg of bromthymol blue, 40 mg of 2,3,5-triphenyltetrazolium chloride, 1,000 ml of distilled water [pH 6.8]) (20). Although variants of both phases can take up triphenyltetrazolium chloride and convert it to formazan and form red colonies, only the primary forms can take up bromthymol blue and thereby produce dark blue colonies surrounded by lighter-colored agar.

Single colonies of each strain were removed from indicator plates and transferred to 500-ml Erlenmeyer flasks containing 200 ml of rich liquid medium (20 g of soybean peptone [Sigma], 5 g of yeast extract [Difco], 1,000 ml of distilled water [pH 6.8]). The cultures were shaken for maximum aeration at different temperatures for 24, 48, 72, and 96 h. At 28°C the liquid cultures reached the stationary phase in 24 to 28 h, at which time the bioluminescence and antibiotic production of *P. luminescens* primary cultures were near maximal; at 18°C maximal levels were reached in 40 to 48 h. Secondary-phase variants usually (but not always) appeared between 48 and 96 h. At low (18°C) temperature, the bacterial growth rate was about half that at 28°C, with the 48-h cultures having passed the logarithmic phase and entered into the stationary phase.

Extraction and analysis of lipids. Lipids were extracted with chloroform-methanol (2:1) as described by Folch et al. (11). The total lipids from two separate experiments were pooled and dissolved in benzene containing 0.01% butylated hydroxytoluene and stored at −20°C until use. Fatty-acid methyl esters were prepared by transesterification in absolute methanol containing 5% HCl in sealed ampules at 80°C for 2.5 h. Fatty-acid methyl esters were separated by gas-liquid chromatography on 10% FFAP coated onto Supelcosyl 100/120 mesh (Supelco, Bellefonte, Pa.) in a 2-m stainless steel column (inside diameter, 2 mm) in a Hitachi model 263-80 gas chromatograph connected to a Hitachi model 263-80 data processor. The column temperature was programmed to rise from

TABLE 1. Relative retention times of fatty acids from lipids of *P. luminescens* separated by gas-liquid chromatography[a]

Sample RT (h)	Standard RT (h)	$RRT_{16:0}$ (h)	Fatty acid
1.76		0.133	10:0
2.00		0.511	i11:0
2.32		0.175	11:0
3.31		0.250	12:0
3.77		0.285	12:1
3.93		0.297	i13:0
4.62		0.350	13:0
5.44	5.55	0.412	i14:0
6.51	6.54	0.493	14:0
7.16		0.542	14:1
7.78		0.589	i15:0
8.28	8.26	0.627	ai15:0
9.19	9.25	0.696	15:0
9.94		0.753	15:1?
10.98	11.07	0.832	i16:0
13.19	13.12	1.000	16:0
14.46		1.096	16:1
15.62		1.184	i17:0
16.85	16.57	1.277	ai17:0
18.43		1.397	17:0
18.45		1.398	?
20.48		1.552	17:1 or cp17:0
22.08		1.673	?
26.15		1.982	18:0
28.90		2.191	18:1
32.57		2.469	ai19:0
38.48		2.917	19:0

[a] RT, retention time; $RRT_{16:0}$, retention time relative to that of 16:0; i, *iso*; ai, *anteiso*; cp, cyclopropane.

140 to 190°C at a rate of 1°C/min. Pooled lipid samples from two separate cultures were used for fatty-acid analysis. Each sample was run in triplicate. For identification of the peaks, the compounds were run isothermally (160°C) and the relative retention times of the methyl esters and of an authentic standard (GLA-110 Standard Mixture; Supelco) were plotted versus the number of carbon atoms in the molecule. The error of determination was less than 5% for major fatty acids (>5% by weight) and 5 to 10% for minor fatty acids (<5% by weight).

Determination of the structural order of liposomes. Liposomes were prepared from bacterial lipids by a modified method of Montaudon et al. (18) by vortexing 1 mg of lipid in 4.0 ml of Tris-HCl buffer (pH 7.4) for 5 min. Visual examination indicated that 100% of the lipid was incorporated into liposomes. The liposomes were sedimented, and 5-doxyl-stearic acid (5-SASL) was added to these liposomes before they were transferred to quartz tubes for electron spin resonance spectrometry (Brucker model ESC-106 ESR spectrometer). Outer hyperfine splitting was determined between 2 and 30°C under medium microwave power (10 mW) and low-magnetic-field modulation (1 mT) as described by Freed (12). Scannings (60 s per spectrum, 2 to 3 h per scan) were made during the heating cycle. Measurements were recorded in 2°C heating increments in which temperature was increased 1.0°C followed by a 2-min stabilization period followed by a 1.0°C increase followed by a 3-min stabilization period.

RESULTS

Fatty-acid composition of total lipids from *P. luminescens* and *X. nematophilus*. In general, the same ca. 25 different fatty acids (even and odd numbered, normal, *iso*, and *anteiso*; fatty acids are represented by a binumeric system in which the first number refers to the chain length and the second number refers to the number of double bonds) emerging between 10:0 and 18:1 were identified in the total lipid extracts of both species. Table 1 contains the retention time values for a standard fatty-acid methyl ester mixture and for a representative sample from *P. luminescens* relative to that for 16:0.

Effect of growth temperature on fatty-acid compositions. Tables 2 and 3 compare the fatty-acid compositions of primary and secondary cultures of *P. luminescens* and *X. nematophilus* grown at different temperatures. Analysis of growth curves showed that cultures grown at 18°C for 48 and 96 h were, with respect to population density, comparable to cultures grown at 28°C for 24 and 72 h, respectively.

TABLE 2. Fatty-acid compositions of total lipids from primary and secondary cultures of *P. luminescens* grown at two different temperatures and for different times

Fatty acid[a]	% (by weight) of total lipids at indicated temp/time (h) in culture type[b]:							
	Primary				Secondary			
	18°C/48	18°C/96	28°C/24	28°C/72	18°C/48	18°C/96	28°C/24	28°C/72
<14:0	2.26	11.30	1.97	−	5.20	9.71	1.03	7.94
14:0	2.64	23.26	5.08	19.57	2.64	23.77	5.50	12.91
14:1	4.04	4.77	2.48	5.97	3.59	1.26	2.76	5.29
15:0	1.94	1.87	1.37	1.43	2.10	1.82	0.45	0.52
16:0	16.83	19.31	34.03	22.05	16.05	19.18	35.76	24.58
16:1	30.04	24.28	23.10	13.15	30.35	24.60	22.18	10.82
17:0	7.32	5.22	1.15	10.19	9.00	6.25	0.60	7.61
cp17:0	4.05	1.96	8.27	16.95	−	5.63	5.42	15.65
18:0	0.26	−	0.92	2.09	0.30	+	0.80	1.32
18:1	30.62	8.03	21.62	8.13	30.80	7.78	25.51	13.36
S/U	0.54	1.64	0.94	2.00	0.54	1.80	0.87	1.85

[a] S, saturated fatty acids; U, unsaturated fatty acids; cp, cyclopropane.
[b] Pooled lipid samples from two separate cultures were used for determinations of fatty-acid compositions.

One of the major differences between primary cultures of *P. luminescens* and *X. nematophilus* was a higher percentage of short-chain fatty acids (<14:0) in the latter at both growth temperatures. The proportions of these fatty acids in the secondary cultures of the two species were lower than in the primary cultures and were quite similar to each other.

Although the fatty-acid compositions in both species were qualitatively similar, there were some characteristic quantitative differences between the percentages of the major fatty acids (e.g., 16:0, 16:1, or 18:1). For example, the proportion of 16:0 was less in primary cultures of *P. luminescens* than in *X. nematophilus* grown at 18°C for 48 h. Similarly, lipids of the primary-phase variant of *P. luminescens* cultured at 18°C for 48 h were richer in 16:1 and 18:1 than in primary cultures of *X. nematophilus*. Increasing the cultivation time from 48 to 96 h

TABLE 3. Fatty-acid compositions of primary and secondary cultures of *X. nematophilus* grown at two different temperatures for different times

Fatty acid[a]	% (by weight) of total lipids at indicated temp/time (h) in culture type[b]:							
	Primary				Secondary			
	18°C/48	18°C/96	28°C/24	28°C/72	18°C/48	18°C/96	28°C/24	28°C/72
<14:0	31.48	24.94	5.54	22.86	9.08	5.94	3.65	8.76
14:0	13.05	17.26	4.40	21.12	6.26	21.46	4.42	20.97
14:1	0.10	4.13	0.86	4.18	0.04	7.85	0.74	3.63
15:0	0.57	0.06	0.95	0.04	0.81	0.28	0.71	0.11
i16:0	−	−	+	−	0.05	0.06	0.03	−
16:0	21.81	22.33	32.93	24.95	23.44	19.13	33.21	26.06
16:1	9.10	11.63	20.33	9.27	29.69	6.22	20.15	7.55
17:0	1.97	−	0.39	0.14	0.49	12.37	0.66	4.07
cp17:0	1.31	8.47	5.53	4.83	0.29	18.27	3.86	15.95
18:0	5.58	0.71	0.04	−	2.17	3.00	1.42	1.28
18:0	15.03	10.47	29.21	12.61	27.68	5.48	31.09	11.62
S/U	3.08	2.48	0.88	2.65	0.73	3.18	0.84	2.69

[a] S, saturated fatty acids; U, unsaturated fatty acids; cp, cyclopropane; i, *iso*.
[b] Pooled lipid samples from two separate cultures were used for determination of fatty-acid composition.

resulted in a decrease in the percentage of the unsaturated fatty acid 18:1 in primary cultures of both species at 18°C, but 16:1 was lowered in only *P. luminescens* at this temperature. Growing the bacteria at the higher temperature (28°C) increased the percentages of 16:0 in both primary cultures. The proportions of unsaturated fatty acids in the two bacterial species responded differently to the increase in growth temperature. The increase in culture temperature from 18 to 28°C reduced the percentages of both 16:1 and 18:1 in *P. luminescens* but increased the proportions of these fatty acids in *X. nematophilus*. Primary cultures of each species responded to prolonged culture time with a reduction of these unsaturated fatty acids (except for 16:1 in *X. nematophilus* at 18°C). At the end of the culture period, the fatty-acid compositions of primary cultures of each species became rather similar, at least with respect to the major saturated and unsaturated fatty acids of even-numbered chain lengths. In *X. nematophilus*, the proportions of fatty acids <14:0 remained high, and there was an increase also in cyclopropane 17:0 at 18°C.

As in the primary-phase variants, the percentages of 14:0 in the secondary cultures of both bacterial species rose with culture time regardless of temperature. In general, the distribution of the major saturated (16:0) and unsaturated (16:1 and 18:1) fatty acids and the patterns of changes with respect to culture time and temperature were similar to those observed for the primary cultures.

Effect of temperature on the structural order of fatty acids in artificial membranes. Because the saturated-to-unsaturated fatty-acid ratio is believed to indicate the "fluidity" of membranes and because this ratio exhibited species-specific changes with increasing growth temperature, we compared the structural order of fatty acids in artificial membranes composed of lipids from primary- and secondary-phase variants of both species. The label selected to determine structural order was 5-SASL, which provides information on the ordering state of fatty acids at the level of the fifth carbonic atom in the bilayer. Lower hyperfine splitting parameter ($2A_{max}$) values indicate less ordered, more fluid structures.

Based on the observed increase in the ratio of saturated to unsaturated fatty acids in primary cultures of *P. luminescens* (Table 2), an increase in the ordering state of fatty acids in cultures grown at 28°C was anticipated. This was not the case; the $2A_{max}$ values were higher at each temperature in cultures grown at 18°C (Fig. 1A). For example, the lipids of *P. luminescens* primary cultures grown at 18°C for 96 h contained a ratio of saturated to unsaturated fatty acids 12% less than that measured for the same species grown at 28°C for 72 h (1.64 versus 2.00); nevertheless, the liposomes prepared from cultures grown at the lower temperature were more ordered (Fig. 1A). In lipids from primary cultures of *X. nematophilus* at similar growth conditions, these ratios were 2.48 versus 2.65, but again the artificial membranes from cultures grown at the lower temperature were more rigid (Fig. 1B). Moreover, lipids from primary cultures of *P. luminescens* grown at 18°C for 96 h were less saturated (saturated-to-unsaturated fatty acid ratio, 1.64) than those from secondary cultures (1.80). Regardless, the former, despite identical growth temperatures and times, were more ordered (Fig. 2A). The same phenomenon also occurred in *X. nematophilus* (ratio of saturated to unsaturated fatty acids, 2.48 versus 3.18) (Fig. 2B).

An interesting difference between the two bacterial strains was that lipid liposomes from primary cultures of *P. luminescens* were more ordered than those from the secondary cultures (Fig. 3A), and this difference was well reflected by the saturated/unsaturated fatty-acid ratios (e.g., at 28°C for 72 h, 2.11 versus 1.67). In contrast to those from *P. luminescens*, membranes prepared from lipids of primary and secondary cultures of *X. nematophilus* exhibited nearly identical fluidities, although membranes from the secondary-phase variant were slightly less ordered (Fig. 3B).

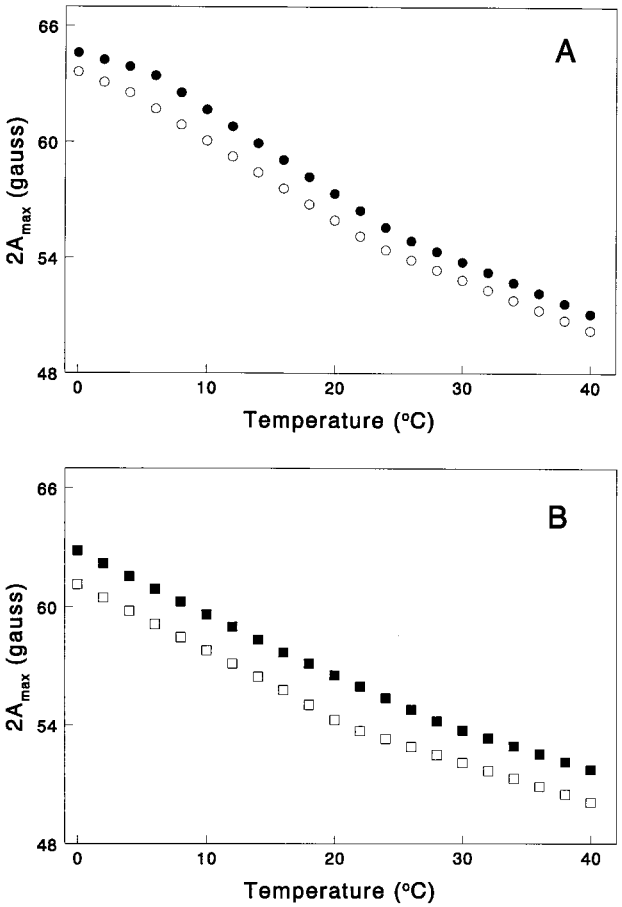

FIG. 1. Temperature dependence of the hyperfine splitting parameters ($2A_{max}$) of 5-SASL embedded in lipid liposomes of primary cultures of *P. luminescens* (A) and *X. nematophilus* (B) grown at 18°C for 96 h (filled symbols) or at 28°C for 72 h (open symbols).

The tendency of primary cultures of *P. luminescens* to form less rigid membranous structures than *X. nematophilus* is evident also upon comparison of the $2A_{max}$ values measured at identical growth temperatures and growth times (Fig. 4). The saturated-to-unsaturated fatty acid ratio in lipids of primary cultures of *P. luminescens* was less than in *X. nematophilus* grown either at 18°C for 96 h (1.64 versus 2.48) or at 28°C for 72 h (2.11 versus 0.87) (Tables 2 and 3), and the former had more-ordered membranous structures than the latter, especially at low measurement temperatures.

DISCUSSION

The results reflect fundamental differences in lipid metabolisms and membrane biophysical properties between *P. luminescens* and *X. nematophilus*. One major difference between the primary forms of the two species is that under identical growth conditions, *X. nematophilus* produces a fatty-acid population that gives rise to less ordered (more fluid) membranous structures, especially at low measuring temperatures, than *P. luminescens*. This greater fluidity of membranes of *X. nematophilus* might be due to an elevated level of short-chain normal

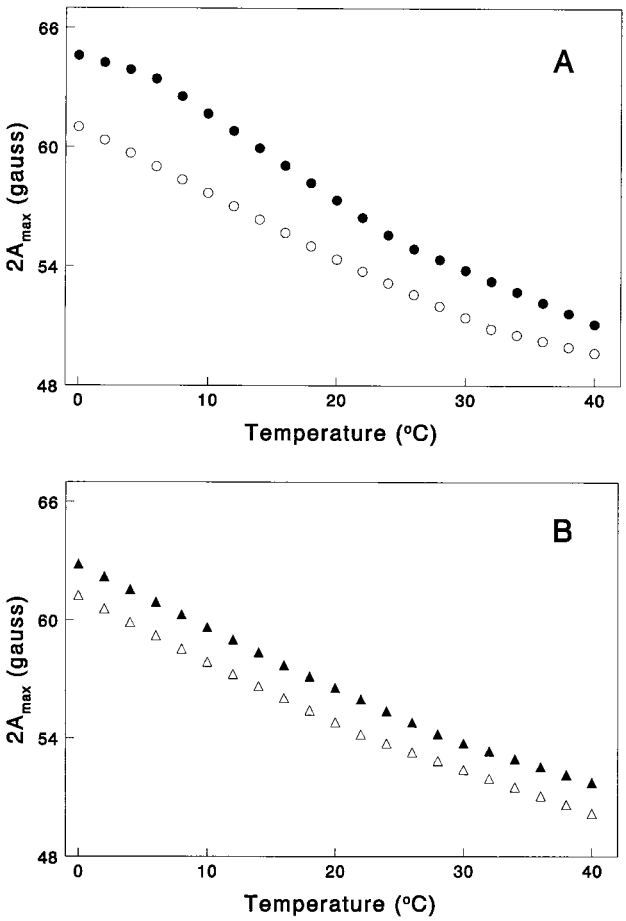

FIG. 2. Temperature dependence of the hyperfine splitting parameters ($2A_{max}$) of 5-SASL embedded in lipid liposomes of primary and secondary cultures of *P. luminescens* (A) and *X. nematophilus* (B) grown at 18°C for 96 h. Filled symbols indicate primary cultures; open symbols indicate secondary cultures.

and branched fatty acids and indicates differences in rates of lipid metabolism between the two species. Short-chain and branched-chain fatty acids are known to increase membrane disorder and fluidity due to their low melting temperatures and greater cross-sectional area, respectively. This result may explain the observations that the *X. nematophilus-Steinernema* complex is more cold tolerant than the *Photorhabdus-Heterorhabditis* complex (4, 17) and may also support the finding that *Xenorhabdus bovienii* tolerates low growth temperatures better than *Photorhabdus* sp. (7).

Both species are similar, however, in their inability to alter the physical properties of their lipid membranes in response to temperature. Both species formed lipid populations giving rise to more-rigid membranous structures when they were grown at lower temperatures, and this fact was not predicted from their fatty-acid compositions. In this respect they differ from *Escherichia coli*, where Sinensky (23) found that the rotational correlation times of 12-SASL measured in extracted lipids were almost identical regardless of the culture temperature. Sinensky termed this phenomenon homeoviscous adaptation (23). The hyperfine splitting parameter ($2A_{max}$) of 5-SASL provides information about the rotational mobility of the probe at the region of the fifth carbonic atom, whereas that of 12-SASL provides information on the hydrophobic core of the bilayer. Although it is possible that differences between our results with 5-SASL and Sinensky's with 12-SASL may result from differences in the probes utilized, additional work with anthroyloxy fatty-acid probes (9a) indicates that the majority of the changes in membrane order occur in the outer half of the bilayer, i.e., the half upon which 5-SASL provides information. In our experiments comparing bacteria grown at two different temperatures, the $2A_{max}$ values were higher at all measurement temperatures (including the culture temperature) in the cells grown at the lower temperature. It is presently unclear whether the difference between *E. coli* and the bacteria investigated in this study results from differences in molecular species composition of the major structural lipids or from some lipid component not considered in this study. For example, measurements of these entomopathogenic bacteria were made on total lipid extracts, which consisted largely of various phospholipids and to a lesser extent neutral lipids; the differences in their ratios might have caused the difference between our and Sinensky's observations. If *X. nematophilus* and *P. luminescens* are similar to *E. coli* and *Pseudomonas fluorescens*, two species in which the levels of phosphatidylethanolamine are rather similar regardless of the growth temperature (8, 16), then the interspecies difference in phosphatidylethanolamine composition between *X. nematophilus* and *P. luminescens* may be minimal. However, because the concentrations of diunsaturated

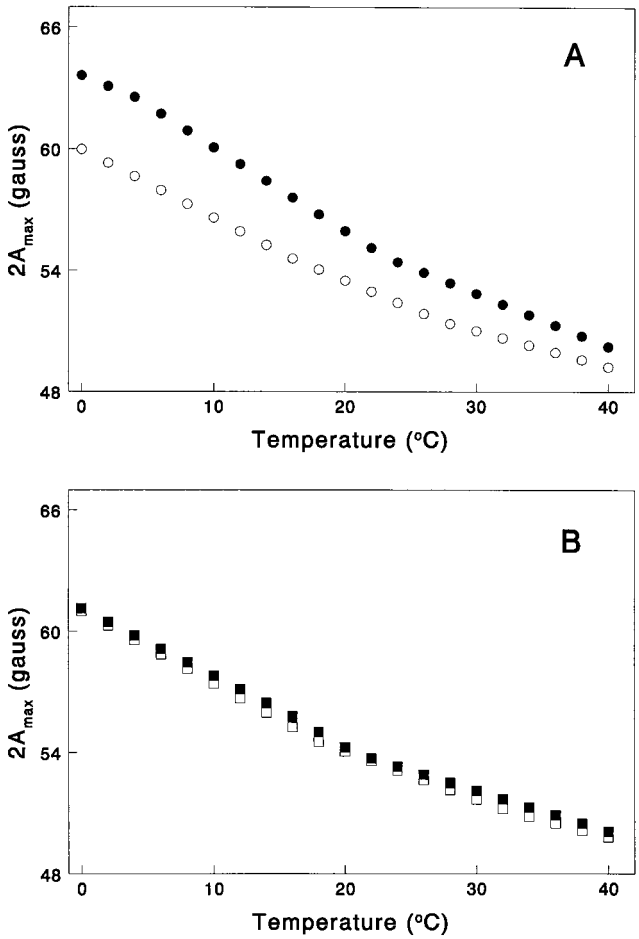

FIG. 3. Temperature dependence of the hyperfine splitting parameters ($2A_{max}$) of 5-SASL embedded in lipid liposomes of primary and secondary cultures of *P. luminescens* (A) and *X. nematophilus* (B) grown at 28°C for 72 h. Filled symbols indicate primary cultures; open symbols indicate secondary cultures.

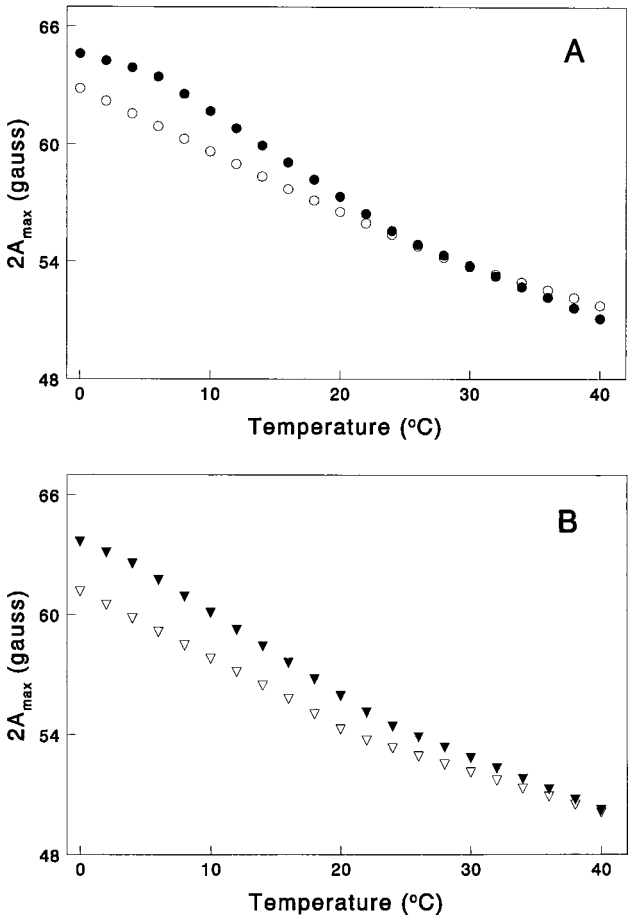

FIG. 4. Comparison of the hyperfine splitting parameters ($2A_{max}$) of 5-SASL embedded in lipid liposomes of primary cultures of *P. luminescens* (filled circles) with those of *X. nematophilus* (open circles) grown at 18°C for 96 h (A) and in lipid liposomes of primary cultures of *P. luminescens* (filled triangles) and *X. nematophilus* (open triangles) grown at 28°C for 72 h (B).

phosphatidylethanolamines and phosphatidylglycerols (which have low thermotropic-phase transition temperatures and larger surface areas than the saturated, monounsaturated, or disaturated species) increase in both the cytoplasmic and outer membranes of *E. coli* at lower growth temperatures (15), future investigations are necessary to determine if the higher $2A_{max}$ values recorded in lipid liposomes of *X. nematophilus* and *P. luminescens* cultured at lower temperatures result from changes in the proportion of one of the constituting lipids and/or accumulation of disaturated species. Interestingly, *Steinernema carpocapsae*, the host of *X. nematophilus*, increased the fluidity of its membrane at reduced temperatures (10).

The results suggest that the transition from the primary- to the secondary-phase variant is accompanied by a detectable decrease in the structural order of fatty acids in artificial membranes. This phenomenon was particularly evident in *P. luminescens* (Fig. 2A and 3A) and occurred to a lesser extent in *X. nematophilus* (Fig. 3B). The difference between the two species might be characteristic and genetically determined.

Because secondary forms yield fewer infective nematode juveniles than primary forms when nematodes are cultured in vitro, the question of whether rigidity of bacterial membranes is a factor in this difference arises. Because membrane permeability depends on membrane fluidity and because only the primary forms release secondary metabolic products (antibiotics, bacteriocins, lipases, proteinases, etc.) (1, 2, 5, 9, 19, 21, 22), it is tempting to speculate that the more rigid membranous structures of the primary-phase variants facilitates this release. This hypothesis may also explain the increase, instead of the expected decrease, of the structural order of fatty acids from *X. nematophilus* and *P. luminescens* cultured at reduced temperatures. The surface areas that phospholipids occupy in the membranes decrease at low temperatures because of reduced thermal motions of the component acyl chains. This reduction might induce the opening of more channels on the cell surface through which the different secondary metabolic products may be released. In this manner the bacteria may maintain in the insect host proper levels of these compounds, which partially protect the bacteria and the cadaver from colonization by other bacterial species, as well as provide digestible food for the nematode host at reduced temperatures. Since *S. carpocapsae* was shown to increase the fluidity of its membrane at lower temperatures (10), the two phenomena may benefit each symbiotic partner. It is indeed remarkable that low temperatures induce an increase in the structural order of the primary forms instead of the expected increase in fluidity.

The more rigid membranous structures of the primary forms might also explain the observation that only the primary forms can be recovered from the intestines of the dauer larvae. Most likely, this difference results from selective digestion rather than from selective uptake of bacteria by nematodes. The membranous structures of the secondary-phase variants, which are more fluid than in the primary-phase variants, may be a factor in susceptibility to digestion; further investigation is necessary to more clearly establish the role of membrane biophysical parameters in this and other facets of the biology of these entomopathogenic bacteria symbiotically associated with nematodes.

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